

INHIBITION KINETICS OF *Sida rhombifolia* L. EXTRACT TOWARD XANTHINE OXIDASE BY ELECTROCHEMICAL METHOD

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ABSTRACT

Sida rhombifolia L. is a traditional medicinal plant that has been known with potential as antigout. The previous research suggested that flavonoids crude extract of *S. rhombifolia* had an inhibitory activity toward xanthine oxidase by 71% and a spectrophotometric measurement showed that the type of flavonoids crude extract inhibition was a competitive inhibition. The purpose of the research was to investigate the type of inhibition kinetic of *S. Rhombifolia*'s ethanol extract by electrochemical method and to compare the measurements of linearity and sensitivity between spectrophotometric and electrochemical methods. The results showed that the yield of *S. Rhombifolia*'s ethanol extract was 9.82% with the inhibitory activity ranging from 13.64% to 82.69% (5.00-200 mg L⁻¹) and IC₅₀ value was 91.15±5.74 mg L⁻¹. Allopurinol as a control showed the inhibitory activity of 15.26-70.95% (0.10-4.00 mg L⁻¹) and IC₅₀ value was 2.45±2.21 mg L⁻¹. Inhibition kinetics of the ethanol extract caused a K_M increase and unchange of V_{MAX}. Based on the data, the type of inhibition kinetics was a competitive inhibition, with an inhibitor affinity (α) value of 3.18. Linearity of xanthine oxidase activity assay by electrochemical and spectrophotometric methods showed the range of 0.01-1.00 mM (R² = 0.978) and 0.05-0.70 mM (R² = 0.977) respectively. The sensitivity of electrochemical method was reported higher (0.95 μA mM⁻¹) than the spectrophotometric method (0.007 min⁻¹).

Keywords: inhibition kinetics; *Sida rhombifolia* L.; xanthine oxidase; electrochemical method

ABSTRAK

Sida rhombifolia L. merupakan tanaman obat yang telah diketahui memiliki potensi sebagai antigout. Penelitian terdahulu menunjukkan bahwa ekstrak flavonoid sidaguri memiliki daya inhibisi terhadap enzim xantina oksidase hingga 71% dan pengukuran secara spektrofotometri menunjukkan ekstrak flavonoid tumbuhan ini mengikuti mekanisme inhibisi kompetitif. Tujuan penelitian ini adalah untuk menentukan tipe kinetika inhibisi dari ekstrak etanol herba sidaguri dengan metode elektrokimia serta membandingkan linearitas dan sensitivitas pengukuran antara metode spektrofotometri dan elektrokimia. Hasil penelitian menghasilkan rendemen ekstrak etanol sebesar 9,82% dengan daya inhibisi mulai dari 13,64% hingga 82,69% (5,00-200 mg L⁻¹) dan IC₅₀ sebesar 91,15±5,74 mg L⁻¹. Adapun allopurinol sebagai kontrol diketahui memiliki daya inhibisi sebesar 15,26-70,95% (0,10-4,00 mg L⁻¹) dan IC₅₀ sebesar 2,45±2,21 mg L⁻¹. Kinetika inhibisi dari ekstrak etanol pada konsentrasi 100 ppm menyebabkan peningkatan nilai K_M (0,187 mM atau meningkat sebesar 68,73%) dari 0,0855 mM menjadi 0,2718 mM, tanpa mengalami perubahan V_{maks}. Berdasarkan hasil tersebut dapat dikatakan tipe kinetika inhibisi yang terjadi mengarah pada tipe kinetika inhibisi kompetitif dengan nilai afinitas inhibitor (α) sebesar 3,18. Metode elektrokimia menunjukkan linearitas pengukuran yang lebih baik dibandingkan metode spektrofotometri, masing-masing pada rentang 0,01-1,00 mM (R² = 0,978) dan 0,05-0,70 mM (R² = 0,977). Sensitivitas metode elektrokimia (0,95 μA mM⁻¹) juga lebih baik dibandingkan metode spektrofotometri (0,007 min⁻¹).

Kata Kunci: kinetika inhibisi; *Sida rhombifolia* L.; xantina oksidase; elektrokimia

INTRODUCTION

Uric acid is final product of purine metabolism. Abnormal conditions of uric acid metabolism will cause

precipitation of sodium urate crystals in joints, a condition termed gout. Gout is caused by an imbalance between the production and excretion of uric acid [1]. Xanthine oxidase (XO) is an enzyme that plays an

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important role in purine metabolism and functions to catalyze hypoxanthine oxidation into xanthine and, xanthine into uric acid. The well known xanthine oxidase inhibitors (XOIs) is allopurinol, which is one option out of the many synthetic drugs used in modern medicine for the treatment of gout [2]. Nevertheless, the use of allopurinol can cause side effects such as allergies, fever, and gastrointestinal disorders. The side effects of synthetic drug use such as allopurinol have prompted people to turn to traditional medicine that utilizes herbs (medicinal herbs) which is safer and more effective.

Sida rhombifolia L. is one of a traditional medicinal plant that has been known with potential as antigout. Iswantini et al. [3] reported that flavonoids crude extract of this plant has a high inhibitory activity of XO, ranging from 48 to 71% (100-800 mg L⁻¹). Not only in a single composition, *Sida rhombifolia* L. potential as antigout in the combined formula has a higher inhibitory activity compared to allopurinol, and this result has been patented and has been granted [4]. In addition to its potential as antigout, the inhibition kinetics determination of *S. Rhombifolia*'s extract as a drug candidate is also one very important thing. The determination of inhibition kinetics type which is formed can subsequently explain the inhibitory mechanism formed and describe the affinity formed between XO enzyme as a target with drug candidate compounds, whether it is temporary (competitive inhibition and uncompetitive inhibition) or permanent (non-competitive inhibition).

Flavonoids crude extract of *S. Rhombifolia* was found out to show the competitive inhibition toward XO [3]. Luteolin and quercetin were reported to follow the competitive inhibition against XO [5]. A noncompetitive inhibition of XO, among others, is indicated by *Fraxinus angustifolia* leaf extract [6] and *Tephrosia purpurea* root extract [7], while the caulerpenyne (CYN) from the extract of *Caulerpa prolifera* was reported to show the uncompetitive inhibition [8].

Method that is commonly and widely used to determine the type of inhibition kinetics is spectrophotometry. Spectrophotometric method is now becoming obsolete, because it is less specific, expensive, highly sensitive to light and is affected by turbidity [9-10]. Therefore, there should be a method which is easier, accurate, rapid and sensitive in the determination of the type of inhibition kinetics. Campanella et al. [11] reported that the electrochemical method was more effective and selective and could overcome the weaknesses of the spectrophotometric method. This method is very promising because of the relatively fast analysis time, which requires inexpensive instrument, accurate, easy miniaturization, and simple operation protocols [12-13]. Electrochemical method for determination of uric acid which have been developed by Arslan [14] using the immobilized uricase with

glutaraldehyde on polyaniline-polypyrrole (pani-ppy) composite film by crosslinking procedure on the surface of a platinum electrode. Ivekovic et al. [15] developed amperometric uric acid biosensor based on uricase with H₂O₂ transducer and electrode modified with Prussian blue.

The purpose of research was to investigate the type of inhibition kinetics of *S. Rhombifolia*'s ethanol extract using an electrochemical method and to compare the measurements of the linearity and sensitivity between electrochemical and spectrophotometric methods.

EXPERIMENTAL SECTION

Materials

Sida rhombifolia L. Plants were collected from its natural habitat in Bogor, East Java, Indonesia.

Instrumentation

Instuments used in this research were an eDAQ potensioestat (Ecoder 410), and data processing software of Echem v 2.1.0. In which an Ag/AgCl electrode, a platinum disk, and carbon paste electrode were used as the reference, counter electrode, and working electrode, respectively, and spectrophotometer.

Procedure

Preparations of carbon paste electrode

Preparations of carbon paste electrode referred to Mirrel et al. [16] with modification. A carbon paste was made from a mixture of graphite powder and paraffin liquid with ratio 2:1 into one end of a glass tubing, and the surface was smoothed and cleaned with a piece of waxed paper. Finally, electrode surface was coated with a dialysis membrane, covered with nylon fibre and tied with parafilm.

Electrochemical measurements

Electrochemical measurements were carried out using an eDAQ potensioestat (Ecoder 410), and data processing software of Echem v 2.1.0. In which an Ag/AgCl electrode, a platinum disk, and carbon paste electrode were used as the reference, counter electrode, and working electrode, respectively. The measurements were performed with an electrolysis cell containing 1.9 mL of basal solution of phosphate buffer 50 mM with predetermined optimum pH. The test solution was deaerated by passing over nitrogen gas unless stated otherwise. The change of currents

response after each addition were observed and recorded.

The inhibitory assay against XO

The inhibitory assay were measured at optimum conditions have been determined previously. The potent inhibitory activity (%) of the crude extract was determined by changes in the XO activity which is analogous to current (I) before and after the addition of extract. Extract concentration varied in the range of 5-200 mg L⁻¹. In addition, the inhibitory activity of allopurinol as a control was also determined by the same procedure.

The Kinetic inhibition assay against XO

The assay was only done on the extract concentration closest to the IC₅₀ value. The type of extract inhibition kinetics was determined by analysis of the Lineweaver-Burk plot. This kinetics study was carried out in the absence and presence of extract with varying concentration of xanthine as the substrate.

Spectrophotometric measurements

Spectrophotometric measurement referred to Tamta et al. [17] which was modified for the determination of velocity assumed as XO activity. Into a tube was inserted 1.9 mL phosphate buffer 50 mM pH 7.5. One milliliter xanthine and 0.1 mL xanthine oxidase 0.1 U/mL were added, and then the solution was incubated at room temperature for 45 min. After the incubation, 1 mL HCl 0.58 M was immediately added to terminate the reaction. The absorbances of the mixed solution were measured at 269.5 nm.

RESULT AND DISCUSSION

Extraction

The dried sample yielded 9.81% of crude extract. Ethanol was used as extracting material because it has two groups with different polarity, i.e. the hydroxyl group which is polar and the alkyl group which is non-polar. Polar and nonpolar compound in the sample are expected to be extracted into the ethanol in the presence of the two groups.

Inhibitory Assay of the Extract against XO

Performance of enzyme activity is generally affected by temperature, pH, and substrate concentration. Therefore, optimization of XO activity was done to study the effects of these parameters on XO activity. Optimized parameters include temperature (25-35 °C), pH [6-9], and substrate concentrations (0.10-1.00 mM). Based on those contours (data not

Table 1. Potent inhibitory effect (%) of several concentration of extract on the activity of xanthine oxidase

No	[<i>S. Rombifolia</i>] (ppm)	Current ΔI_{pa} (μA)	Inhibitory Effect (%)
1	0	1.173	0
2	5	1.013	13.640
3	10	0.833	28.986
4	20	0.810	30.946
5	50	0.747	36.317
6	100	0.513	56.266
7	200	0.203	82.694

Table 2. Potent inhibitory effect of several concentration of allopurinol on the activity of xanthine oxidase

No	[allopurinol] (ppm)	Current ΔI_{pa} (μA)	Inhibitory Effect (%)
1	0	1.160	0
2	0.1	0.983	15.259
3	0.5	0.867	25.259
4	1	0.783	32.500
5	2	0.673	41.983
6	3	0.487	58.017
7	4	0.337	70.948

shown), the optimum conditions for each of the parameter temperature, pH and xanthine concentration was 30 °C, pH 7.5, and 1.00 mM. Kong et al. [18] was reported the condition of similar pH. As for the other different measurement conditions, it was reported by CeCngiz et al. [8], that is, at a temperature of 37 °C and pH 9.0. The differences in the optimum conditions for the measurement of temperature and substrate concentration are caused by different methods, tools and measurement time.

The potential of ethanol crude extract as antigout was determined by observing the inhibitory activity of the extract at various concentrations against XO activity. Analyses of these various concentrations were aimed to investigate the effects of the increase in the concentration on the inhibitory activity. In addition, concentration variation will facilitate and provide flexibility to the selection of the extract concentration to be used as a candidate on kinetics inhibition assay.

The results of extract inhibition effect can be seen in Table 1, which shows that there is an increase in inhibition effect in line with the increase in extract concentration. Linear relationship between the inhibition effect and extract concentration is shown by the decrease of current (I) which is analogous to XO activity after the addition of extract at various concentrations. The *S. Rombifolia*'s ethanol extract is known to have inhibitory activity (%) by 13.64% to 82.69% (5-200 mg L⁻¹). Iswantini et al. [3] previously reported that the inhibitory assay of flavonoid crude extract from this plant using the spectrophotometric

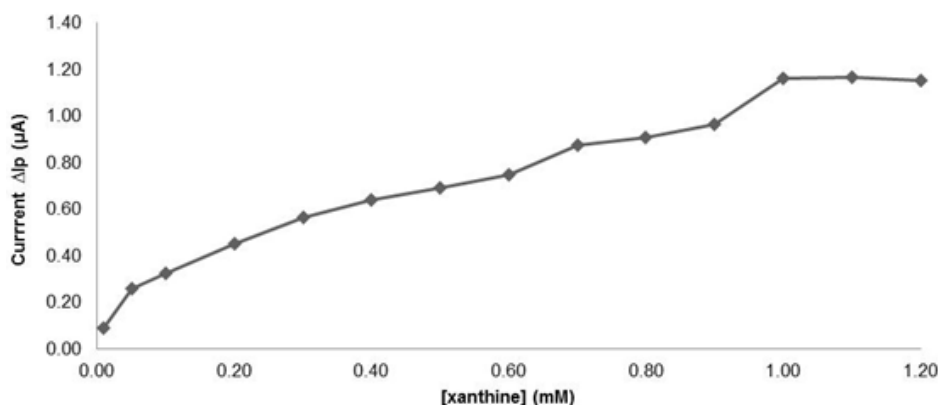


Fig 1. Relationships between xanthine concentration and current responses as XO activity

method showed an inhibitory activity of 48-70.71% at a concentration of 100-800 mg L⁻¹.

The measurable differences of the inhibition effect in this study and Iswantini et al. [3] were presumably because the spectrophotometric measurement which is strongly influenced by the concentration of test solution so that the response becomes less good. It can be indicated that the electrochemical method can respond to the inhibitory activity of extracts better compared to the spectrophotometric method. According to Thuong et al. [19] a compound is said to be active if it has a value of IC₅₀ less than 100 mg L⁻¹. The results showed the IC₅₀ values of extract of 91.15±5.74 mg L⁻¹.

Inhibitory Assay of Allopurinol against XO

As comparison in this study, the inhibitory activity and IC₅₀ of allopurinol (Table 2) were also determined. The analysis showed that allopurinol had a stronger inhibitory activity with IC₅₀ value of 2.45±2.21 mg L⁻¹. Other studies on allopurinol inhibitory activity indicated that IC₅₀ allopurinol which is varied, among others, 6.1 μg mL⁻¹ [20-21], 3.74 μg mL⁻¹ [22] and 4.29 μg mL⁻¹ [23].

This indicated that the electrochemical method carried out in this study gave a value of IC₅₀ which was relatively small compared with the spectrophotometric method reported by Umamaheswari et al. [20] and Azmi et al. [22]. However, several other studies have also reported the IC₅₀ value which was relatively smaller at 0.6 μg mL⁻¹ [24]. IC₅₀ values in some of these studies may be different because it is influenced by differences in observing conditions [25].

Linearity and Sensitivity Measurement

Effect of substrate concentration on the XO activity can be determined by measuring the activity of XO with varying concentrations of xanthine as the substrate. Fig. 1 which is identical to the Michaelis-Menten curve shows the relationship xanthine concentration and XO activity.

Reaction catalyzed by the enzyme was occurred in two phases. First, if the substrate concentration is low, the enzyme active sites did not fully interact to the substrate. Second, when the substrate concentration increases, the active sites is bound entirely by substrate and at this time the enzyme has been working at full capacity so that the addition of substrate does not affect XO activity [10]. When the concentration of xanthine is less than 1.00 mM, the reaction is in the first phase and when the xanthine concentration is greater than 1.00 mM, XO activity is in the second phase.

Fig. 2 shows the linear relationship between substrate concentration and current responses as XO activity by the electrochemical method in the range of 0.01-1.00 mM. Linear range obtained in the electrochemical measurement is better when compared with the results obtained with the spectrophotometric method. The spectrophotometric measurement was carried out at the maximum wavelength (λ_{max}) 269.50 nm and showed a linear measurement in the substrate concentration range of 0.05-0.70 mM (Fig. 3). Spectrophotometric measurement gave high absorbance (>2) at the substrate concentration >0.70 mM in the calibration curve of xanthine, that indicates the less accuracy and precision of measurement. Therefore, the electrochemical method can respond analyte more specifically and in the wider concentration range than the spectrophotometric method.

Sensitivity is the ratio of change in response per unit change in analyte concentration [26]. Sensitivity can be determined based on the slope value of the calibration curve [27]. The result showed that the electrochemical and spectrophotometric methods each gave a sensitivity value of 0.95 mA mM⁻¹ and 0.007 min⁻¹. The higher of sensitivity value indicates that with a small change of concentration can provide a significant change in response [28]. Thus the electrochemical method was indicated to provide a better sensitivity than the spectrophotometric method in

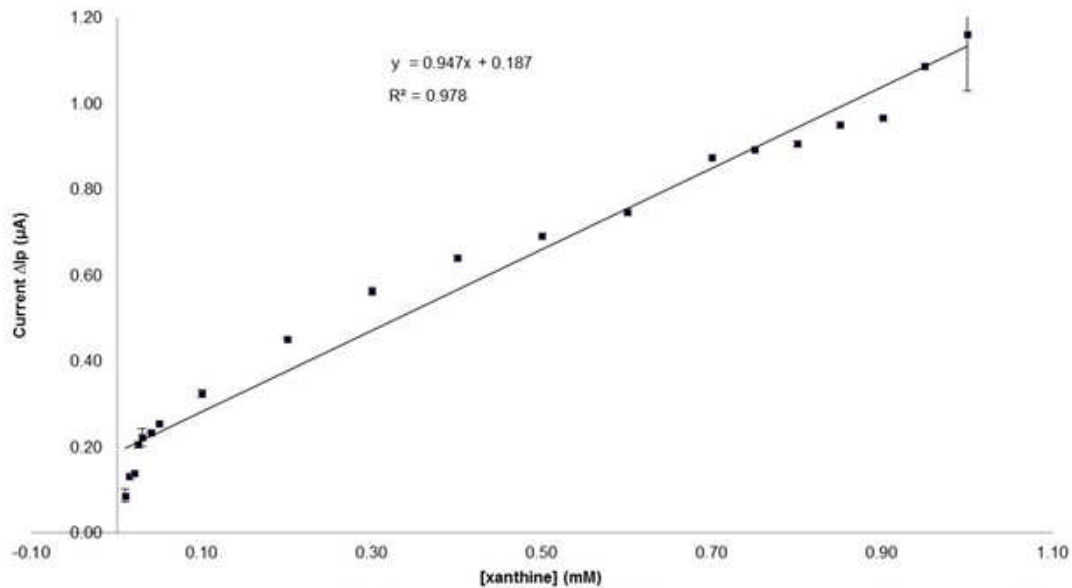


Fig 2. Linearity between xanthine and XO activity (V_{max}) by electrochemical method

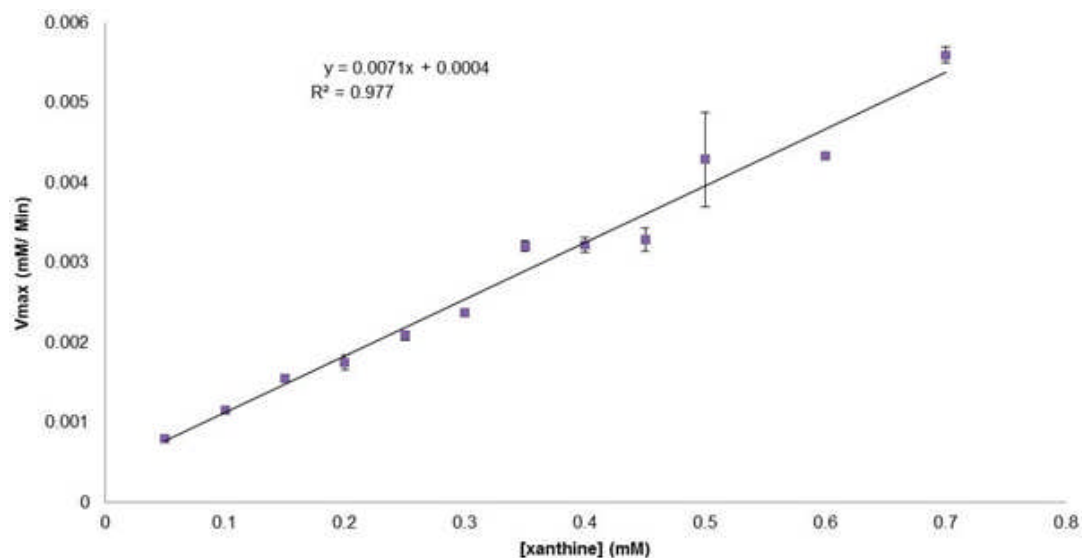


Fig 3. Linearity between xanthine and xanthine oxidase activity (V_{max}) by spectrophotometric method

determining the velocity of enzymatic reactions that form the basis for determining the type of inhibition kinetics. The other researches by electrochemical methods were reported to give varying sensitivity values. A smaller sensitivity value, among others, was reported at $0.55 \mu\text{A mM}^{-1}$ [29] and $0.062 \mu\text{A mM}^{-1}$ [30]. While the higher of sensitivity values was reported by Zhao et al. [31] of $29.5 \mu\text{A mM}^{-1}$. The electrochemical method can also be reported more effectively than the spectrophotometric method in determining the type of inhibition kinetics because electrochemical method requires a shorter analysis time, higher sensitivity and the amount of material required is also relatively less than the spectrophotometric method.

Kinetic Properties of XO

Concentration of selected extracts in inhibition kinetics assay was 100 ppm. The selection was based on its high inhibitory activity (>50%) and the closest concentration of IC_{50} extract values that had been obtained previously. Qualitative analysis of the enzymatic reaction is generally carried out by Michaelis-Menten curve, but the curve is very difficult to determine the value of K_M and V_{max} . Therefore, in this study the determination of K_M dan V_{max} values was carried according to Lineweaver-Burk plot (Fig. 4).

Determination of the inhibition kinetics type was done by determining changes in the value of the

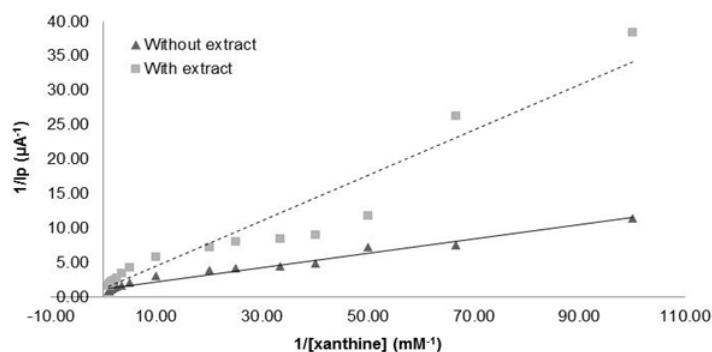


Fig 4. Lineweaver-Burk plot of XO inhibition with various concentrations of xanthine, addition of 100 ppm of ethanol extract of *Sida rhombifolia* L., without ethanol extract and Lineweaver-Burk transformed data were plotted and followed by linear regression of the point

Michaelis-Menten constant (K_M) and maximum velocity (V_{max}) which is analogous to a maximum current (I_{max}). Based on the analysis of Lineweaver-Burk plot (Fig. 4), it resulted a significant change of K_M value and very small V_{max} change. The kinetic pattern that forms after the addition of extracts as inhibitors resulted in an increase in K_M from 0.0855 mM to 0.2718 mM, an increase of 68.73%. The I_{max} value decreased from 0.82338 to 0.82325 and the small decrease in the I_{max} value could be assumed there was no change [32]. Based on the results it can be concluded that ethanol crude extract of this plant leads to the type of competitive inhibition kinetics as can be concluded from similar V_{max} and different K_M values [33].

Electrochemical measurements give a smaller value of K_M and a higher value of V_{Max} which were previously obtained on spectrophotometric measurements by Iswantini et al. [3]. K_M is a measure of substrate affinity for the enzyme. The smaller the K_M value obtained on electrochemical method shows that this method can measure the substrate affinity of the enzyme even at relatively lower concentrations than in the spectrophotometric measurements.

The inhibitor affinity value (α) of the enzyme can be determined by calculating the ratio between the value of K_M with inhibitors and K_M values without inhibitor. The inhibitor affinity (α) of extract was 3.18. These conditions indicate the existence of strong competition between the extract and the substrate to bind with the active sites of enzyme. The Competitive inhibition kinetics of *S. Rhombifolia*'s ethanol extract found in this study reinforce the previous research by Iswantini et al. [3] which explains a competitive inhibition of *S. Rhombifolia*'s flavonoids crude extract with smaller α value of this research, that is, 2.32.

CONCLUSION

The *S. Rhombifolia*'s ethanol extract has a potential as XO inhibitor as indicated by its inhibitory

activity up to 82.69% (200 ppm) and $IC_{50} < 100 \text{ mg L}^{-1}$. Inhibition kinetics of the ethanol extract caused a change of K_M and similar of V_{max} . Thus, the type of inhibition kinetics leads a competitive inhibition, with an inhibitor affinity (α) value of 3.18. Electrochemical and spectrophotometric measurements showed a linear region of each measurement in the range of 0.01-1.00 mM and 0.05-0.70 mM. The sensitivity of electrochemical method in velocity measurement was reported to be higher ($0.95 \text{ } \mu\text{A mM}^{-1}$) than the spectrophotometric method (0.007 min^{-1}). Electrochemical method provides fast analysis time, high sensitivity, easy operation, and inexpensive method in determining the type of inhibition kinetics.

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